DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

THE DIVISION OF AIDS

NATIONAL INSTITUTE OF ALLERGY & INFECTIOUS DISEASES

NATIONAL INSTITUTES OF HEALTH

and

COLLABORATING INVESTIGATORS

CHIRON QUANTIPLEX® HIV RNA 2.0 ASSAY
HIV-RNA Quantitation by Branched DNA (bDNA)

I. PRINCIPLE

The Quantiplex® HIV-RNA Assay is a sandwich nucleic acid hybridization procedure for the quantification of HIV type 1 RNA in human plasma. HIV is first concentrated from plasma via centrifugation. After HIV genomic RNA is liberated from virions, its capture to a microwell is mediated by a set of specific, synthetic oligonucleotide target probes. Another set of target probes hybridize to the viral RNA and the pre-amplifier probes. The pre-amplifier probes hybridize to the branched DNA (bDNA) amplifiers. Specifically, the two sets of target probes bind to different regions of the pol gene of the viral RNA.

Multiple copies of an alkaline phosphatase-labeled probe are hybridized to the immobilized complex to amplify the signal. Detection is achieved by incubating the complex with a chemiluminescent substrate and measuring the light emission generated by the bound alkaline phosphatase. Light emission is directly proportional to the amount of HIV RNA present in each sample, and results are recorded as luminescent counts by a plate luminometer. A standard curve is defined by light emission from standards with known concentrations of recombinant bacteriophage. Concentrations of HIV-RNA in specimens are determined from the curve.

II. SPECIMEN REQUIREMENTS

The bDNA assay has been optimized for 1.0 mL plasma collected in EDTA. Acid Citrate Dextrose (ACD) is also an acceptable anticoagulant. Heparin may result in depressed quantitation and should not be used.

No special preparation of the patient is required prior to specimen collection. However, proper specimen handling is extremely important.

Store whole blood at room temperature before separating plasma from cells; **Do Not refrigerate.**

Use standard procedures to remove plasma from cells within 4 to 6 hours of collection. Do not clarify plasma by filtration. If the plasma specimen is not to be tested within 30 minutes of separation, store it at -60^{0}C to -80^{0}C in a sterile, screw-capped tube. It is convenient to freeze specimens in accurately measured 1 mL aliquots. Avoid repeated thawing and freezing.

III. REAGENTS

Chiron Quantiplex® HIV RNA 2.0 Assay Kit:

1. Box 1 (shipped refrigerated at 2-8^{0}C) Component:

a. HIV Wash A (buffered solution with sodium azide and antimicrobial preservatives)

b. HIV Wash D (buffered solution with sodium azide and antimicrobial preservatives)

c. Plate Sealers

d. HIV Capture Wells (polystyrene microwell strips coated with synthetic oligonucleotides)

e. HIV lysis diluent (buffered solution containing protein and synthetic oligonucleotides with sodium azide and antimicrobial preservatives)

f. HIV lysis reagent (stabilized proteinase K solution)

g. HIV label diluent (buffered solution with protein stabilizers; with sodium azide and antimicrobial preservatives)

h. HIV substrate (chemiluminescent substrate - Lumi-Phos® Plus)

i. HIV Substrate Enhancer (contains 0.05% sodium azide and 0.05% Proclin™ 300 as preservatives)

j. HIV Pre-Amplifier (buffered solution containing protein™ and synthetic oligonucleotides with sodium azide and Proclin 300 as preservatives)

k. HIV Amplifier (buffered solution with protein stabilizers and synthetic oligonucleotide molecules including branched DNA with sodium azide and antimicrobial preservatives)

l. HIV Standards Diluent (buffered solution containing protein and synthetic oligonucleotides with sodium azide and antimicrobial preservatives)

m. HIV Bead Suspension (buffered solution containing inert polystyrene beads with 0.05% sodium azide and Proclin™ 300 as preservative)

n. HIV Target Probes (synthetic oligonucleotides in water with sodium azide and antimicrobial preservatives)

2. Box 2 (shipped frozen on dry ice) Component:

a. HIV Label Probe (enzyme-labeled synthetic oligonucleotide in buffer solution with sodium azide and antimicrobial preservatives)

b. HIV Positive Control (human plasma containing beta propriolactone-treated HIV with sodium azide and antimicrobial preservatives)

c. HIV Negative Control (human plasma nonreactive for HIV-RNA and nonreactive for HIV antibody, with sodium azide and antimicrobial preservatives)

d. HIV Standard A (human plasma with a known concentration of recombinant single-stranded DNA with sodium azide and antimicrobial preservatives)

e. HIV Standard B (human plasma with a known concentration of recombinant single-stranded DNA with sodium azide and antimicrobial preservatives)

f. HIV Standard C (human plasma with a known concentration of recombinant single-stranded DNA with sodium azide and antimicrobial preservatives)

g. HIV Standard D (human plasma with a known concentration of recombinant single-stranded DNA with sodium azide and antimicrobial preservatives)

3. VQA Control provided by the Virology Quality Assurance Laboratory (VQA)

Storage of reagents:

Observe manufacturer's outdates.

Box 1 is shipped refrigerated and should be stored refrigerated (2-8^{0}C) upon receipt.

Box 2 is shipped frozen on dry ice. Store Box 2 and its entire contents frozen (-60 to -80^{0}C)

VQA reagents should be stored in accordance with VQA instructions

IV. EQUIPMENT AND SUPPLIES

Laminar flow biosafety cabinet (class II)
Gloves
Lab coat
Adjustable micropipettors (1000 µL, 200 µL, 20 µL)
Sterile, disposable tips, with and without aerosol resistant barriers

Multichannel pipette, 12-channel, with 15 µL, 150 µL, and 200 µL volumes
Disposable reagent reservoirs for multichannel pipette
Vortex mixer
Refrigerated centrifuge (RCF = 23,500 x g), 24 tube fixed angle rotor (45)
Dry heat block (53^0C)
Incubator or waterbath (37^0C)
Eight welled aspirator, connected to 4L vacuum flask and vacuum pump
Chiron® heater (53^0C) with 8 x 12 metal microwell holder
Chiron® luminometer (37^0C ± 2.5^0C)
Screw-cap, conical, sterile, 1.5 mL microcentrifuge tubes with o-rings
Sterile, disposable plastic 15 mL and 50 mL tubes
Sterile, disposable plastic 6 mL tubes
Blank microwells
Sterile, serological pipettes
Household bleach (5.25% sodium hypochlorite or equivalent)

V. PROCEDURE

The Quantiplex® HIV-RNA Assay procedure consists of two parts: (1) specimen preparation and (2) the assay procedure itself. Specimens are prepared by centrifugation, which concentrates the virus into a pellet. Pellets can be prepared in advance and stored for up to 2 weeks at -60^0C to minus 80^0C. It is convenient and cost effective to prepare specimens in advance and test them in a full or half plate, batch mode. If pelleted specimens are not to be tested immediately *(*within 10 minutes*)* after preparation, they must be frozen.

Each kit contains sufficient reagents and materials for one 96-well run, consisting of 4 standards, 2 controls, and 84 patient specimens, to be tested. When assaying partial plate, prepare only half volumes of working reagents, and store unused reagents and kit components as directed. Fill unused plate positions with blank microwells.

Using fewer than 48 wells at one time is not recommended.

A. Critical parameters

1. Bring assay components to indicated temperature before use.

2. Thaw frozen reagents immediately before use by placing in cool tap water. Vortex briefly.

3. Prepare working reagents immediately before use as instructed in the assay procedure.

4. Return all assay components to recommended storage conditions after use.

5. Add all reagents and specimens to HIV capture wells by touching the pipette tip to the wall near the midpoint of the well, above the surface of the fluid in the well.

6. For best results, do not break apart HIV capture well strips into small segments, as they may adhere to the plate sealer and spill their contents upon its removal.

7. HIV capture wells must be sealed securely during incubations to prevent evaporation. Before each incubation, press a new plate sealer very firmly onto the tops of the wells, using a sweeping motion of the palm of the hand or a flat object.

8. When removing the plate sealer following incubations, use care not to pull the HIV capture wells out of the plate holder. Use one hand to hold the wells in the plate holder while slowly and carefully peeling back the plate sealer with the other hand.

9. When vortexing materials, mix with at least five complete pulses.

10. Assay all Standards, Negative control, and Positive control in duplicate.

11. Before adding materials to HIV capture wells, position the metal plate holder with its notches on the left.

B. Preparation of specimens and controls

1. Pre-chill the centrifuge and rotor to 4^0C.

2. In a Biological Safety Class II cabinet, prepare a vacuum flask containing 100 mL of household bleach and a secondary trap attached to a vacuum source. Connect a plastic aspirating device to the vacuum flask.

3. Thaw one rotor's complement of specimens and the HIV Positive, Negative and VQA Controls in cool tap water for approximately 10 minutes. As soon as the specimens and controls are thawed, proceed to the next step. Do not leave the thawed specimens in tap water for an extended period of time.

4. Transfer 1 mL of specimen or control aliquots into labeled 1.5 mL screw-capped tubes.

5. Mix HIV Bead Suspension by inversion. Add 50 µL HIV Bead Suspension to each 1 mL specimen or control.

6. Cap tubes and centrifuge at 23,500 x g for 1 hour at 2 to 8^0C.

7. In a biological safety cabinet, open the rotor and carefully remove tubes.

8. Immediately aspirate the supernatant from each control and specimen using a plastic aspirating device equipped with a clean, sterile, disposable 200 µL plastic tip. Use a new tip for each specimen and control. Do not disturb the pellet, the location of which is indicated by the colored microparticles. Do not aspirate the pellet to dryness; leave 15 to 20 µL of supernatant.

9. Immediately (within 10 minutes) proceed with the assay procedure or freeze the virus pellets at -60^{0}C to -80^{0}C. Pelleted virus is stable for up to 2 weeks when frozen.

C. Assay procedure--day 1

1. Preheat the Chiron® Heater to 53 ± 0.5^{0}C for at least 30 minutes. (The Chiron® Heater may be left on for an extended period of time between assay runs.)

2. Remove HIV Lysis Diluent, HIV Standards Diluent, HIV Lysis Reagent, HIV Target Probes, HIV Capture Wells, HIV Wash A and D, and Plate Sealers from BOX 1. Warm HIV Lysis Diluent and HIV Standards Diluent to 37^{0}C for 10 to 15 minutes before use. Mix HIV Lysis Diluent and Standards Diluent by inversion.

3. Prepare HIV Specimen Working Reagent by pipetting 20 mL of HIV Lysis Diluent into a sterile 50 mL tube and adding 140 µL of HIV Target Probes and 2.4 mL of HIV Lysis Reagent (full plate). For half plates, add 10 mL of lysis diluent, 1.2 mL of lysis reagent, and 70 µL of HIV target probe into the sterile tube. Mix by inversion and maintain at room temperature (15^{0}C-30^{0}C) for up to 4 hours. See figure 3A, 3B, 3C and 3D for Reagent Preparation Chart.

4. Prepare HIV Standards Working Reagent by pipetting 1.5 mL of HIV Standards Diluent into a sterile 6 mL tube and adding 15 µL of HIV Target Probes and 270 µL of HIV Lysis Reagent. Mix by inversion and maintain at room temperature (15^{0}C-30^{0}C) for up to 4 hours.

5. Remove one rotor's complement of pelleted specimens or controls (in groups of 24, staggered 10 minutes apart) from the freezer and add 220 µL of Specimen Working Reagent to each tube. Cap the tubes and vortex for 10 seconds. **NOTE: If pellets were frozen, thaw at room temperature up to 5 minutes prior to adding HIV Specimen Working Reagent.**

6. Incubate tubes in a heat block at 53^{0}C for 20 ± 2 minutes.

7. During the incubation, set up the microwell plate as shown in Figure 1 or Figure 2.

Figure 1
Microwell Full Plate Setup

STD A	**STD A**	**STD B**	**STD B**	**STD C**	**STD C**	**STD D**	**STD D**	**NEG**	**NEG**	**POS**	**POS**

VQA	VQA	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC

Figure 2
Half Plate Setup

STD A	**STD A**	**STD B**	**STD B**	**STD C**	**STD C**	**STD D**	**STD D**	**NEG**	**NEG**	**POS**	**POS**
VQA	VQA	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC
SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC	SPEC

a. Allow the HIV Capture Well pouch to reach room temperature before opening. Remove HIV Capture wells from their pouch and place them in the metal 8x12 microwell holder provided with the Chiron Heater. Break the plastic tabs at the end of each strip. (If only 48 wells are needed, return the remaining 48 wells to the pouch, seal with tape, and store at 2^0C- 8^0C. Fill the lower half of the plate holder with blank microwells. Do not distribute reagents to blank wells.)

b. Record positions of HIV Standards, HIV Controls, VQA Control and patient specimens on Plate Map.

Figure 3A
HIV Specimen Working Reagent Preparation

Number of HIV Capture Well Strip	**HIV Lysis Diluent**	**HIV Lysis Reagent**	**HIV Target Probes**
4 (1/2 plate)	10 mL	1.2 mL	70 µL
8 (full plate)	20 mL	2.4 mL	140 µL

Figure 3B
HIV Standards Working Reagent Preparation

Number of HIV Capture Well Strip	HIV Lysis Diluent	HIV Lysis Reagent	HIV Target Probes
4 (1/2 plate) or 8 (full)	1.5 mL	270 µL	15 µL

Figure 3C
HIV Label Working Reagent Preparation

Number of HIV Capture Well Strip	HIV Label Diluent	HIV Label Probe
4 (1/2 plate)	3 mL	50 µL
8 (full plate)	6 mL	100 µL

Figure 3D
HIV Substrate Working Reagent

Number of HIV Capture Well Strip	HIV Substrate	HIV Substrate Enhancer
4 (1/2 plate)	1.5 mL	1.5 mL
8 (full plate)	3 mL	3 mL

8. Following the incubation, remove the tubes from the heat block and vortex for 10 seconds.

9. Hold tubes at room temperature for 5 minutes.

10. During the final heat block incubation, thaw HIV Standards A, B, C, and D at room temperature.

11. Transfer 200 µL of patient specimens, and controls into the appropriate HIV Capture Wells.

12. After all specimens and controls are loaded into the HIV Capture Wells, add 150 µL of HIV Standards Working Reagent to those wells designated for HIV Kit Standards.

13. Vortex Kit HIV Standards A, B, C, and D and add 50 µL of each Standard to the appropriate wells.

14. Seal the plate with a plate sealer, pressing firmly to avoid vapor leaks. Ensure that every microwell if fully covered.

15. Immediately incubate the plate in the Chiron® Heater at 53 ± 0.5^{0}C for 16 to 18 hours. Refer to the Chiron® Heater Operator's Manual for proper setup and use.

    Note: Immediately vortex pellets and incubate at 53^{0}C after addition of Specimen Working Reagent.

D. Assay procedure--day 2

16. Warm HIV Wash A and HIV Wash D to room temperature (15^{0}C-30^{0}C).

17. Warm HIV Pre-Amplifier, HIV Amplifier and HIV Label Diluent at 37^{0}C for at least 10 to 15 minutes until solutions are homogeneous. Mix by inversion and use within 2 hours.

    Note: If solutions are not homogeneous, continue heating until reagent is in solution.

18. Prepare a 4 L vacuum flask with 100 mL of household bleach. Connect the multiwell aspirator to the vacuum flask.

19. Carefully remove the plate from the heater. After an overnight incubation, the HIV Capture Wells may stick in the lid of the Chiron® Heater. If this occurs, use a spatula to dislodge the plate and remove carefully. Set the plate on an uncovered bench top to cool for 10 ± 1 minutes. Carefully observe wells and note any well from which fluid has evaporated.

20. Carefully peel back the Plate Sealer with one hand, using the other hand to hold the HIV Capture Wells in place. Discard Plate Sealer.

21. Using an 8-channel microwell vacuum manifold or equivalent, aspirate the entire contents of each well into the vacuum flask containing the disinfectant solution.

22. Using the multichannel pipette or bottle top dispenser, fill each HIV Capture Well with approximately 400 µL of HIV Wash A. Then, completely remove HIV Wash A from every well by aspirating contents into the vacuum flask. Repeat this wash cycle a second time; be certain to thoroughly aspirate the second wash.

    Note: Do not allow HIV Capture Wells to remain dry for more than 5 minutes.

23. Using the multi-channel pipette and reagent reservoir, add 50 µL of HIV Pre-Amplifier to each HIV Capture Well. Deliver the solution to the side of the well. Cover the plate with a plate sealer as previously described.

24. Incubate the plate in the Chiron® Heater at 53^{0}C for 30 ± 3 minutes.

25. Carefully remove the plate from the heater. Set the plate on a bare bench top or other cool surface at room temperature for 10 ±1 minutes. Observe and record any well from which fluid has evaporated.

26. Carefully remove the plate sealer.

27. Repeat step 21 and 22 as previously described.

28. Using the multi-channel pipette and reagent reservoir, add 50 µL of HIV Amplifier to each HIV capture well. Deliver the solution to the side of the well. Seal the plate with a fresh plate sealer as previously described.

29. Incubate the plate in the Chiron® Heater at 53 ± 0.5 C for 30 ± 3 minutes.

30. During the incubation, thaw HIV Label Probe at room temperature and vortex to mix.

31. Repeat step 25 as previously described.

32. Prepare HIV Label Working Reagent by pipetting 6 mL of HIV Label Diluent into a sterile 15 mL tube and adding 100 µL of HIV Label Probe. Mix by inversion and maintain at room temperature. Use within 10 minutes. Return remaining Label Probe to -60 to -80^{0}C.

33. Carefully remove the plate sealer and discard. Thoroughly aspirate the contents of all wells, and wash wells twice with HIV Wash A as described in step 21 and 22.

34. Using the multi-channel pipette and reagent reservoir, add 50 µL of HIV Label Working Reagent to each HIV Capture Well. Securely seal the plate with a new Plate Sealer as described previously. Incubate in Chiron® Heater at 53^{0}C for 15 ± 2 minutes.

35. During this incubation period, program the Chiron® Luminometer to read the plate as instructed in the Chiron® Luminometer Operator's Manual. Users of the Chiron® Date Management System should prepare it to receive data according to the software instructions.

36. During the label incubation, bring the HIV Substrate and HIV Substrate Enhancer to room temperature.

37. Remove the plate from the Chiron® Heater and place it on an uncovered bench top at room temperature for 10 ± 1 minutes. Carefully remove and discard Plate Sealer. Thoroughly aspirate contents from wells, and wash wells twice with HIV Wash A as described previously.

38. Aspirate the wells thoroughly. Use the multichannel pipette or a second bottle top washer to wash wells 3 times with HIV Wash D in the same manner described previously. Be certain to remove all of HIV Wash D after the final wash step.

39. Prepare HIV Substrate Working Reagent by pipetting 3 mL of HIV Substrate into a sterile-packaged polypropylene tube and adding 3 mL of HIV Substrate Enhancer. Mix by inversion. Use within 10 minutes. Solution will be cloudy.

40. Add 50 µL of HIV Substrate Working Reagent to each well using the multi-channel pipette. Securely seal the plate with a Plate Sealer.

41. Immediately insert the plate into the Chiron® Luminometer, aligning the notches in the plate with the pins in the loading tray. Incubate the plate for 30 minutes at 37 ± 2.5^{0}C. The Luminometer will automatically read the relative light units in each of the wells at the end of the 30 minute incubation period.

    Note: When processing more than one plate, begin incubating the second plate 5 minutes after the first. Incubate the second plate in the 37^{0}C dry incubator for 25 minutes and transfer it to the Chiron® Luminometer to incubate at 37^{0}C for the final 5 minutes prior to reading.

42. When the plate reading is completed, proceed as instructed in the Chiron® Data Management Software Manual.

VI. CALCULATION AND INTERPRETATION OF RESULTS

The Chiron® luminometer reports results in relative luminescence (RL) units, which are a measure of the amount of light emitted from each microwell. Light emission is directly proportional to the number of HIV-RNA copies present in each patient specimen.

To quantitate HIV-RNA, the mean RL value of each specimen is compared to the standard curve. The final result is reported in HIV-RNA copies x 10^3/mL.

The Chiron® Data Management System software automatically calculates average relative luminescence values and %CV (coefficient of variation) for HIV Standards, HIV Controls, and patient specimens, plots the standard curve, calculates the HIV-RNA copies x10^3/mL for each patient specimen, and prints out a report.

A. Interpretation of Results

1. The cutoff value for the Quantiplex® HIV-RNA Assay is 0.5 RNA copies x 10^3/mL.

2. Specimens with values above or equal to (≥) 0.5 RNA copies x 10^3/mL contain HIV-RNA in the quantity indicated.

3. Specimen with values less than (<) 0.5 RNA copies x 10^3/mL are below the detection limit of the assay.

4. Specimen with values greater than (>) the assigned value of HIV Standard A are above the upper limit of the Standard Curve and must be diluted to obtain a quantitative value.

VII. QUALITY CONTROL AND EXPECTED VALUES

1. The assay control and standards supplied with the test kit must be included with each run, regardless of the number of specimens tested or capture wells used. HIV value assignments and the expected quantitation of the Positive control are shown in the Product Insert Supplement provided with each kit.

2. If the quantification of the HIV Positive Control is outside of the range listed in the supplement, the entire run is invalid and must be repeated.

3. The negative control must be below the cutoff, if it quantifies, the run is invalid and must be repeated.

4. If the relative luminescence (RL) units for the kit standards do not meet the following criteria: "RLU STD A > RLU STD B > RLU STD C > RLU STD D", the run is invalid and must be repeated.

5. Similarly, VQA standards must yield expected results or the entire assay may need to be repeated.

VII. REFERENCES

1. Chiron® Product insert (Quantiplex® HIV-RNA), L6170 rev 5.0, June 1994

2. Personal communications: Chiron® Technical Services